

A hypoallergenic variant of Der p 1 as a candidate for mite allergy vaccines

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Background: Recombinant hypoallergens that display reduced allergenicity but retain T-cell reactivity represent promising candidates to improve the safety and efficacy of allergen-specific vaccines or immunotherapy.

Objective: The current study reports the immunologic characterization of a hypoallergenic variant of the major mite allergen Der p 1.

Methods: The recombinant proform of Der p 1 (ProDer p 1) was expressed in *Escherichia coli* (ProDer p 1 *coli*), purified and characterized at the level of its secondary structure, and IgE and T-cell reactivities. Moreover, the prophylactic potential of ProDer p 1 *coli* vaccinations was evaluated in a murine Der p 1 sensitization model.

Results: After purification and refolding, ProDer p 1 *coli* remained aggregated with a higher β -sheet content and altered Der p 1 conformational epitopes compared with the correctly folded monomeric ProDer p 1 produced in Chinese hamster ovary cells. Both ProDer p 1 forms were able to retain the Der p 1-specific T-cell reactivity but direct ELISA, competitive inhibition, and rat basophil leukemia assays clearly showed that ProDer p 1 *coli* displays a very weak IgE reactivity. Mice vaccinations with aggregated ProDer p 1 adjuvanted with alum induced a T_H1-biased immune response that prevented the subsequent allergic response after Der p 1 sensitization and airway challenge with aerosolized mite extracts. Furthermore, ProDer p 1 *coli* treatment inhibited the development of airway eosinophilia and airway hyperresponsiveness to inhaled methacholine.

Conclusion: Aggregated forms of Der p 1 could represent hypoallergens suitable for the prevention of mite allergy.

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Key words: Der p 1, recombinant hypoallergen, aggregates, prophylactic vaccinations

Sensitization to allergens of the house dust mite *Dermatophagoides pteronyssinus* is one of the most prevalent forms of atopy in industrialized countries. Amongst the approximately 20 mite allergens, Der p 1 is one of the most immunodominant antigens because the prevalence of anti-Der p 1 IgE antibodies in patients with mite allergy is higher than 80%.¹ The potent allergenicity of Der p 1 is not fully understood, but recent data provided evidence for a correlation between the proteolytic activity of Der p 1 and the exacerbation of CD4⁺-T_H2 cell activation.²⁻⁷

Although clinical studies demonstrated the efficacy of specific immunotherapy, the only allergen-specific treatment of type I allergy, the administration of increasing doses of crude allergen extracts to patients may induce severe and life-threatening IgE-mediated anaphylactic side effects.⁸ Consequently, the generation of recombinant hypoallergenic allergens that display low or no IgE binding activity but still retain T-cell epitopes to evoke T-cell responses would increase the safety of immunotherapy with a reduced risk of anaphylactic reactions.⁹

We successfully produced at high yield a recombinant precursor form of Der p 1, secreted as ProDer p 1, in insect or Chinese hamster ovary (CHO) cells as well as in *Pichia pastoris*.¹⁰⁻¹² Because ProDer p 1 was shown to be enzymatically inactive, we could consider that ProDer p 1 is less allergenic than Der p 1. However, the recent elucidations of the 3-dimensional structure of recombinant ProDer p 1¹³ and mature Der p 1¹⁴ highlighted common structure features between ProDer p 1 and the native allergen. Consequently, ProDer p 1 could likely maintain the overall repertoire of the conformational IgE-binding epitopes of Der p 1 except for those blocked by the prosequence.¹⁵

The current study investigated the allergenicity and immunogenicity of ProDer p 1 expressed in *Escherichia coli*. Our data clearly showed that the prokaryotic expression of ProDer p 1 leads to the formation of a hypoallergenic variant of Der p 1 suitable for the prevention of house dust mite allergy.

METHODS

Antigens

Natural Der p 1 was purified from whole *D pteronyssinus* culture by immunoadfinity chromatography,¹⁶ and purified ProDer p 1 expressed in CHO cells (ProDer p 1 CHO) or produced in *P pastoris* were obtained as previously described.^{11,12}

Expression of ProDer p 1 in *E coli*

The ProDer p 1 coding cassette from the pNIV4846 plasmid¹¹ was amplified by PCR using the following primers: 5'GGGGCTAGCCGCGCCA GTCCTAATAGACC3' (*NheI* restriction site in boldface and underlined,

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Abbreviations used

AHR:	Airway hyperresponsiveness
ATR:	Attenuated total reflectance
BAL:	Bronchoalveolar lavage
CHO:	Chinese hamster ovary
FTIR:	Fourier Transform Infrared
Penh:	Enhanced pause
ProDer p 1:	Recombinant proform of Der p 1
RBL:	Rat basophil leukemia

forward) and 5'GGGGCGGCCGCCAGGATCACCACGTACGGG3' (*NotI* restriction site in bold and underlined, reverse). The amplified fragment was digested with *NheI*-*NotI* to generate a 920-bp fragment and introduced into the pStaby1 expression vector (Delphi Genetics, Gosselies, Belgium) restricted with the same enzymes. The resulting plasmid contained the ProDer p 1 cassette upstream to a (His)₆ sequence tag. This DNA construct was transformed by electroporation into CYS21 *E coli* cells (Delphi Genetics). The presence and identity of the ProDer p 1 cDNA was verified by DNA sequencing. Finally, the recombinant plasmid purified from 1 CYS21 clone was transformed into the production SE1 *E coli* cells (Delphi Genetics) according to the manual instructions. Recombinant ProDer p 1 (ProDer p 1 *coli*) was expressed at 37°C after addition of 1 mmol/L isopropyl-thiogalactoside (Duchefa, Haarlem, The Netherlands) for 2 hours at an OD_{600 nm} of 0.6 to 0.8.

Purification of ProDer p 1 from *E coli*

The induced bacteria were collected by centrifugation (15 minutes at 9500g), resuspended in 40 mL cold TRIS buffer 50 mmol/L, pH 7.5, supplemented with Aprotinin 1 mmol/L (Sigma, Bornem, Belgium) and 4-(2-aminophenyl)benzenesulfonyl fluoride hydrochloride 1 mmol/L (MP Biomedicals Solon, Ohio), and lysed by 2 passages through a cell disrupter (Cell D; Constant, Low March, United Kingdom) at 1800 bars. The cell lysate was centrifuged for 20 minutes at 3000g to isolate the inclusion bodies. The pellet was washed with TRIS buffer 50 mmol/L, pH 7.5, Triton X-100 1%, followed by 3 washing steps to remove the detergent. ProDer p 1 was subsequently extracted overnight at 4°C with 40 mL TRIS buffer 50 mmol/L, 300 mmol/L NaCl, and 6 mol/L urea, pH 7.5. After ultracentrifugation (45 minutes, 149,000g), the supernatant of extraction was applied at 3 mL/min on a Ni²⁺ chelate high-performance column (2.6 × 6 cm; GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated with the extraction buffer. The column was washed with the starting buffer. Protein elution proceeded by stepwise increasing imidazole concentration in the buffer (from 0 to 400 mmol/L). Fractions containing purified ProDer p 1 (elution with 200 mmol/L imidazole) were pooled. ProDer p 1 *coli* was renatured by a 3-step dialysis to remove urea (from 6 mol/L to 2 mol/L urea, from 2 mol/L to PBS + 500 mmol/L L-arginine, from PBS + 500 mmol/L L-arginine to PBS). The recombinant allergen was concentrated by ultrafiltration (regenerated cellulose ultrafiltration membranes, cutoff 10 kD; Millipore, Billerica, Mass) and stored at -20°C.

Size exclusion chromatography

ProDer p 1 CHO or *coli* was applied onto a Superdex 200-HR column (10 × 300 mm; GE Healthcare Bio-Sciences), equilibrated in a PBS buffer at pH 7.3. The Superdex 200-HR column was calibrated by using blue dextran (2,000 kD), bovine IgG (150 kD), BSA (67 kD), ovalbumin (43 kD), and cytochrome C (14 kD).

Determination of secondary structure contents by attenuated total reflectance-Fourier Transform Infrared

Purified protein samples (1-3 µg) were microdialyzed against 1 mmol/L HEPES, pH 7.2, and spread on a micro-attenuated total reflectance (ATR) crystal for measurement in attenuated total reflexion mode.¹⁷ Spectra (64 acquisitions averaged, 2 cm⁻¹ resolution) were recorded on a Bruker IFS-55 Fourier-Transform Infrared (FTIR) spectrophotometer equipped with a liquid

nitrogen-cooled Mercury Cadmium Telluride detector. Acquired spectra were corrected for water vapor and amino-acid side-chain contribution in the amide I and II bands and smoothed with a 4 cm⁻¹-width Gaussian. For secondary structure comparisons, deuterium-saturated nitrogen was continuously flushed in the chamber containing the ATR crystal, and spectra were recorded after reaching a plateau phase in the amide II exchange kinetics (60 minutes).

Der p 1 ELISA

Recombinant ProDer p 1 CHO or *coli* were detected with an ELISA kit by using Der p 1 specific mAbs 5H8 and 4C1 (Indoor Biotechnologies, Charlottesville, Va). The Der p 1 standard (UVA 93/03) used in the assay was at a concentration of 2.5 µg/mL.

IgE-binding activity

Direct ELISA as well as IgE inhibition assays were performed as previously described.¹⁸ The specific anti-*D pteronyssinus* IgE values (ImmunoCAP; Phadia, Uppsala, Sweden) of sera from patients with mite allergy were above the upper cutoff value of 100 kU/L.

T-cell reactivity

Heparinized venous blood samples were collected from 3 adult volunteers of both sexes with house dust mite allergy. All patients had chronic symptoms and positive skin prick test results to house dust mite extract (HAL, Haarlem, The Netherlands). Human PBMCs (2 × 10⁵ cells/well) were cultured in quadruplicate in the absence or presence of Der p 1, ProDer p 1 CHO, or ProDer p 1 *coli* in the presence of anti-IL-10 receptor mAb to block the bioactivity of endogenously produced IL-10¹⁹ (R&D Systems, Abingdon, United Kingdom). After 5 days, proliferative responses were measured by [³H]thymidine incorporation and expressed as stimulation indexes.

Rat basophil leukemia cell mediator-release assay

Rat basophil leukemia cells RBL-SX-38 (a kind gift of Professor Kinet, Beth Israel Deaconess Medical Center, Boston, Mass), expressing human FcεRI, were sensitized with 4 sera from a patient with *D pteronyssinus* allergy (IgE value > 100 kU/L, 1/10 dilution) for 17 hours. Triggering of RBL cells was induced by adding various concentrations (1-0.0001 µg/mL) purified Der p 1, ProDer p 1 CHO, or *coli*. As a control, cells were also incubated in the absence of allergen to measure spontaneous release. Total release was obtained by adding 0.5% Triton X-100 to the medium. The release of β-hexosaminidase was analyzed by incubating supernatants (50 µL) with 50 µL *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (2 mmol/L in 0.2 mol/L citric acid buffer, pH 4.5; Sigma, Bornem, Belgium) for 3 hours at 37°C. The reaction was stopped by adding 150 µL 1 mol/L TRIS-HCl, pH 9, and the absorbance at 405 nm was measured. Results were expressed as percentage of the total release minus the spontaneous release.

Animals

Female Balb/c mice (6 weeks old) were obtained from Harlan (Horst, The Netherlands). Animal care and experimental procedures were carried out in accordance with local institutional guidelines (laboratory license n°LA 1500474).

Immunization protocols

Groups of 7 Balb/C mice were immunized 3 times at 2-week intervals with 5 µg ProDer p 1 CHO or *coli* adjuvanted with alum (ratio allergen/adjuvant 1/20, intraperitoneal injection). As a control, 1 group of mice was immunized with saline alone. Mice were bled 7 days after the last injections (day 35), and sera were collected.

Three weeks after the last vaccination, animals were sensitized at weekly intervals for 3 weeks with 5 µg natural Der p 1 formulated with alum (ratio allergen/adjuvant 1/20, intraperitoneal injection). To induce airway inflammation, mice were airway allergen challenged 13 days after the last sensitization (day 76) by exposition to aerosolized crude *D pteronyssinus* extract (containing 10 µg/mL Der p 1) over a 30-minute period for 4 consecutive days. For each mouse, serum, bronchoalveolar lavage fluid (BALF), and spleen were collected on day 80.

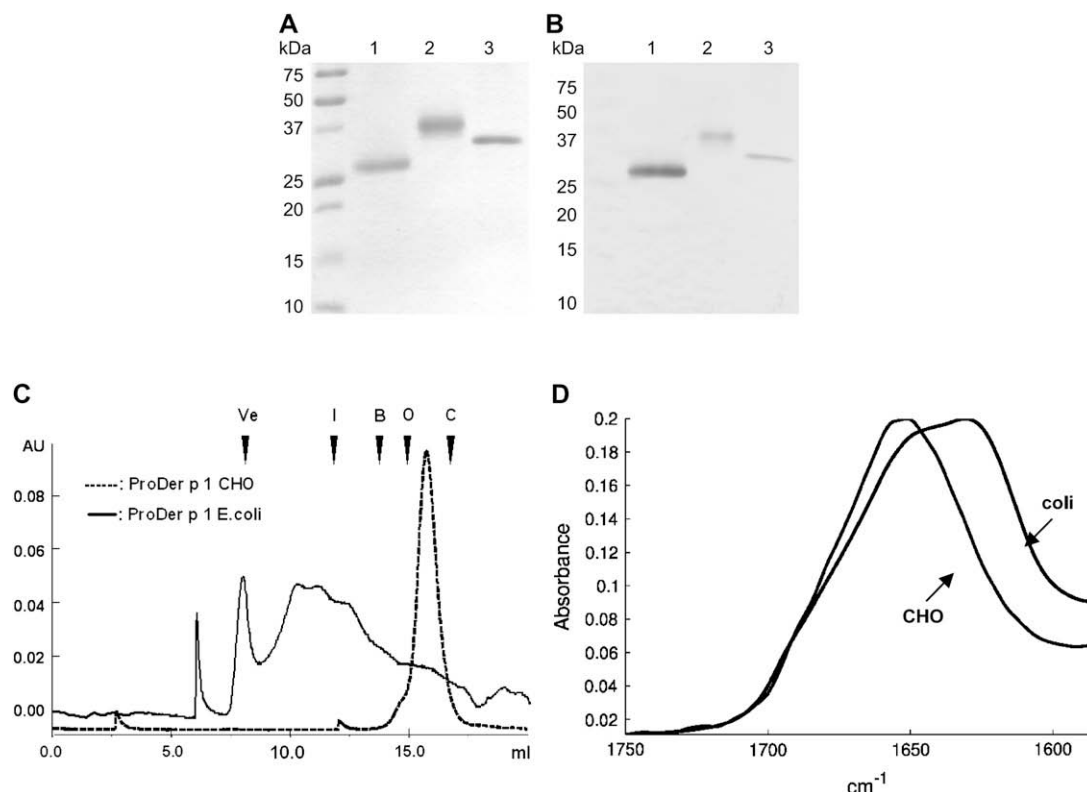


FIG 1. Characterization of ProDer p 1 *coli*. Coomassie-stained SDS-PAGE (**A**) and Western blotting (**B**) of natural Der p 1 (lane 1), recombinant ProDer p 1 CHO (lane 2), and ProDer p 1 *coli* (lane 3) incubated with anti-Der p 1 polyclonal antibodies. **C**, Gel filtration of ProDer p 1 *coli* or CHO. The Superdex 200-HR column was calibrated by using blue dextran (Ve, 2.000 kD), bovine IgG (I, 150 kD), BSA (B, 67 kD), ovalbumin (O, 43 kD), and cytochrome C (C, 14 kD). **D**, Infrared absorbance spectra of ProDer p 1 *coli* or CHO. Spectra are normalized at full scale on the amide I band. AU, Absorbance unit.

Measurement of allergen-specific response

Anti-ProDer p 1 IgG₁, IgG_{2a}, IgE antibody assays, BAL analysis, Der p 1-specific T-cell proliferative responses, and IFN- γ and IL-5 production in supernatant from restimulated splenocytes were performed as previously described.¹⁸ For lymphoproliferative assays, cells were restimulated with ProDer p 1 produced in *P. pastoris*.¹²

Measurement of airway hyperresponsiveness

One day after the last allergen challenge, airway responsiveness was measured via whole-body plethysmography (model PLY 3211; Buxco Electronics, Troy, NY) in unrestrained mice after challenge with increasing doses of methacholine (Sigma, Bornem, Belgium) as described elsewhere.²⁰ The average enhanced pause (Penh) above a saline challenge baseline value was compared between groups. This dimensionless parameter reflects the total pulmonary airflow in mice and is a function of the sum of the airflows in the upper and lower respiratory tracts during a respiratory cycle.

Statistical analysis

Group data were compared by using the Student *t* test. Differences were considered significant for *P* values <.05.

RESULTS

Expression and purification of ProDer p 1 *coli*

ProDer p 1 was expressed in *E. coli* as a C-terminally hexahistidine-tagged protein that accumulated as inclusion bodies. The recombinant allergen, solubilized in urea, was purified to

homogeneity by nickel affinity chromatography under denaturing conditions. Purified ProDer p 1 *coli* was subsequently refolded by successive dialysis against decreasing concentrations of urea and using L-arginine, a well known additive that can prevent aggregation during refolding. On SDS-PAGE, purified ProDer p 1 *coli* migrated as a single band of 35 kD, whereas natural Der p 1 ran at 29 kD (Fig 1, A). Both bands were recognized with murine polyclonal antibodies raised to ProDer p 1 CHO (Fig 1, B). ProDer p 1 CHO migrated as an immunoreactive species of 38 kD corresponding to a glycosylated form of ProDer p 1.¹¹

ProDer p 1 *coli* adopts a folding typical of aggregated proteins

Three different approaches were used to determine whether ProDer p 1 *coli* displays the common folding of ProDer p 1 and then the overall structure of Der p 1: size exclusion chromatography, infrared spectroscopy, and Der p 1 ELISA. The Superdex 200 elution profile of ProDer p 1 CHO clearly showed that this recombinant allergen is monomeric, whereas the vast majority of ProDer p 1 *coli* was polymerized into soluble larger oligomeric structures with an apparent molecular weight ranging from 120 kD to higher than 600 kD (Fig 1, C).

The secondary structure content of ProDer p 1 CHO, estimated by ATR-FTIR spectral analysis and using a curve-fitting procedure of the deuterated amide I band, reveals 28% α -helices,

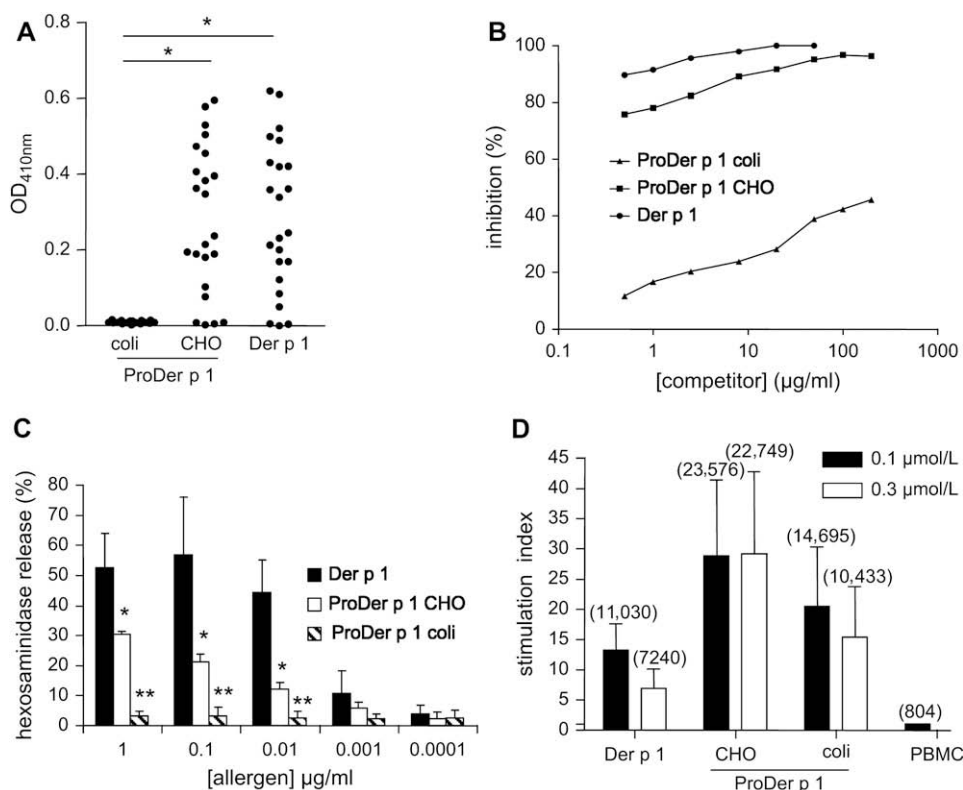


FIG 2. ProDer p 1 *coli* displays very weak IgE reactivity but retains the Der p 1 T-cell reactivity. **A**, IgE reactivity of natural Der p 1 and recombinant ProDer p 1 *coli* or CHO evaluated in direct ELISA and using ImmunoCAP positive sera to *D pteronyssinus* (N = 23). **P* < .001 compared with Der p 1 or ProDer p 1 CHO. **B**, ELISA inhibition assays. The binding of human IgE to natural Der p 1 was inhibited by using natural Der p 1 (●), recombinant ProDer p 1 *coli* (▲), or CHO (■). The % inhibition is shown as 100 – (percentage of IgE binding in the presence of inhibitor / percentage of IgE binding in the absence of inhibitor). **C**, Mediator release of natural Der p 1 or recombinant ProDer p 1 *coli* or CHO cells with humanized RBL SX-38 cell line. Results were expressed as percentage of the total release of β-hexosaminidase minus the spontaneous release ± SD. **P* < .001 compared with Der p 1. ***P* < .001 compared with Der p 1 or ProDer p 1 CHO. **D**, Induction of T-cell proliferation. PBMCs from individual patients (n = 3) were stimulated with Der p 1, ProDer p 1 CHO, and ProDer p 1 *coli* (concentration as indicated). Results are expressed as the means ± SEMs of quadruplicate stimulation index. Mean absolute thymidine counts are indicated between brackets.

39% β-sheets, 18% β-turns, and 15% random coil (Fig 1, D). In contrast, the shape of the amide I band is modified in ProDer p 1 *coli*, resulting in a shoulder at 1620 cm⁻¹ in bands at frequencies lower than 1630 cm⁻¹, characteristic of many aggregated proteins, with an apparent increase in antiparallel β pleated sheets.²¹ The secondary structure proportions were 23%, 46%, 16%, and 15% for α-helices, β-sheets, β-turns, and random coil, respectively.

Finally, both ProDer p 1 forms were compared in a sandwich ELISA assay by using 2 anti-Der p 1 mAbs that recognize conformational epitopes of the allergen. Contrary to ProDer p 1 CHO, ProDer p 1 *coli* could not be detected in this ELISA assay, confirming that this recombinant form does not adopt the appropriate Der p 1 folding (data not shown).

ProDer p 1 *coli* displays a very weak IgE reactivity

In a direct ELISA using 23 sera from house dust mite-sensitized patients, ProDer p 1 *coli* was shown to display a very weak IgE-binding reactivity compared with natural Der p 1 or ProDer p 1 CHO (*P* < .0001; Fig 2, A). The IgE reactivity of ProDer p 1 *coli* was next analyzed by competitive inhibition assays using

pooled sera (N = 20) from patients preincubated with various concentrations of Der p 1 or ProDer p 1 forms (Fig 2, B). As previously observed,^{11,12} natural Der p 1 was able to inhibit IgE-binding to coated Der p 1 more efficiently than ProDer p 1 CHO used at the same concentrations. The inhibition capacity of ProDer p 1 *coli* was drastically reduced compared with that of ProDer p 1 CHO.

Finally, mediator release assays performed with a RBL cell line expressing human FcεRI, passively sensitized with sera from patients with *D pteronyssinus* allergy and stimulated with both ProDer p 1 forms or Der p 1, indicated that reduced IgE-binding capacity of ProDer p 1 *coli* correlated with lower allergenic activity (Fig 2, C).

ProDer p 1 *coli* retains T-cell reactivity

To demonstrate that ProDer p 1 *coli* is able to maintain its ability to stimulate Der p 1-specific T cells, PBMCs from *D pteronyssinus*-sensitized patients were stimulated *in vitro* with Der p 1 or ProDer p 1 CHO or *coli*. Similarly to Der p 1 or ProDer p 1 CHO, ProDer p 1 *coli* was able to restimulate the PBMCs from mite-sensitized patients (Fig 2, D), suggesting that ProDer p 1 *coli* retained the relevant Der p 1-specific T-cell epitopes.

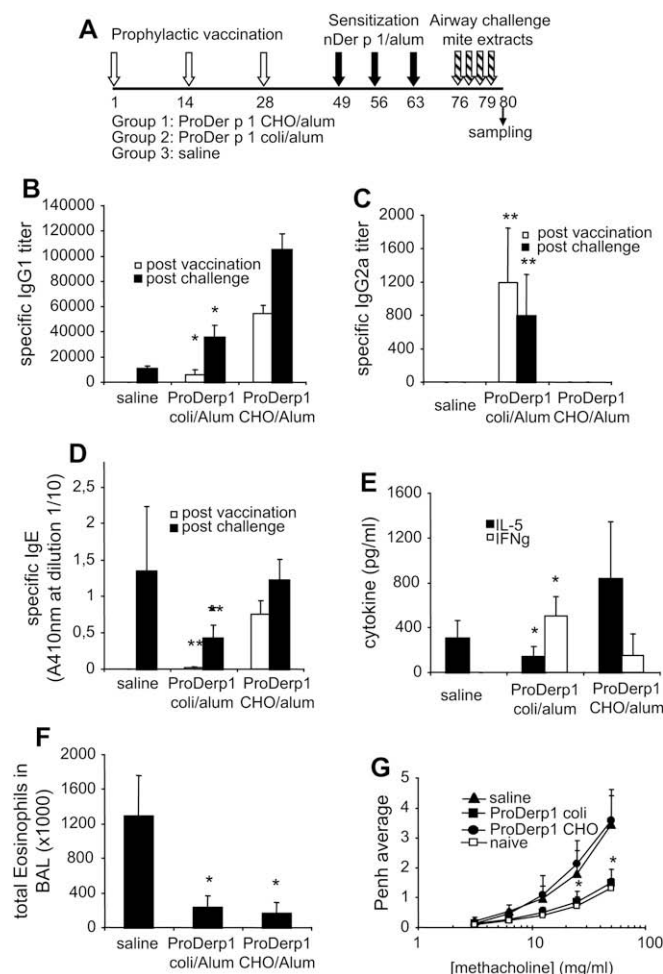


FIG 3. Prevention of experimental house dust mite allergy by vaccinations with recombinant ProDer p1 *coli*. Mice ($n = 7$) were vaccinated with saline (PBS), recombinant ProDer p1 *coli* or CHO, sensitized to natural Der p 1, and challenged (A). Antibody titers (IgG₁, B; IgG_{2a}, C; IgE, D), splenocytes cytokine production (E), airway eosinophilia (F), and airway hyperresponsiveness (G) were plotted as means $\pm$ SDs. One representative experiment out 3 is shown. * $P < .05$, ** $P < .001$ compared with saline-vaccinated and ProDer p1 CHO-vaccinated mice (C-E, G), with saline-vaccinated mice (F), and with ProDer p1 CHO-vaccinated mice (B). nDer p 1, Natural Der p 1.

Evaluation of the prophylactic potential of ProDer p 1 *coli* in a Der p 1 sensitization murine model

Balb/C mice were immunized with ProDer p1 *coli* or CHO adjuvanted with alum. As control, animals were vaccinated with saline. Pretreated mice were subsequently sensitized with Der p 1 adsorbed to alum and exposed to aerosolized house dust mite extract to provoke airway inflammation. Mice immunized by 3 injections of ProDer p1 CHO/alum produced high titers of anti-ProDer p1 IgG₁ (Fig 3, B) and large amounts of specific IgE antibodies (Fig 3, D). In contrast, the anti-ProDer p1 IgG_{2a} response was absent (Fig 3, C). ProDer p1 *coli* was shown to be less immunogenic than ProDer p1 CHO ($P < .05$ for IgG₁ titer comparison, Fig 3, B). However, ProDer p1 *coli* treatment induced significant production of anti-ProDer p1 IgG_{2a} ($P < .001$; Fig 3, C) and, strikingly, prevented the development of specific IgE antibodies ($P < .001$; Fig 3, D). The subsequent Der p 1 sensitizations and aerosol challenges were shown to increase

the specific IgG₁ and IgE titers in both ProDer p1-vaccinated groups but did not alter the ProDer p1 *coli*-induced IgG_{2a} response (Fig 3, B-D). However, this induction of the allergen-specific IgE response was strikingly less marked in ProDer p1 *coli*-vaccinated mice (Fig 3, D). The prevention of the allergen-specific IgE antibodies together with the induction of a specific IgG_{2a} response clearly suggested that ProDer p1 *coli* induced a T_H1-biased specific response, even in the presence of a pro-T_H2 adjuvant such as alum.

Splenocytes from control or ProDer p1-vaccinated mice were stimulated with ProDer p1 produced in *P. pastoris*, and supernatants from cultured stimulated lymphocytes were harvested and assayed for IFN- γ and IL-5. A typical T_H2 cytokine profile characterized with high production of IL-5 and low or no secretion of IFN- γ was elicited in Der p1-sensitized mice previously treated or not with ProDer p1 CHO (Fig 3, E; $P > .05$). Again, the T_H1 bias of the immune response induced by ProDer p1 *coli* was confirmed because animals vaccinated with this recombinant allergen secreted respectively, large and low amounts of IFN- γ and IL-5 on restimulation (Fig 3, E; $P < .05$).

Animals immunized with Der p1 and challenged with aerosolized house dust mite extracts developed an important airway eosinophilia in BAL fluids (Fig 3, F). In contrast, pretreatments with both ProDer p1 forms significantly decreased the eosinophil influx in the airways, indicating that the prevention of airway eosinophilia by ProDer p1 vaccinations is independent of the structure of the allergen ($P < .05$). These results demonstrated that a immune response induced by ProDer p1 *coli* has beneficial effects on allergen-induced airway inflammation.

Challenge with house dust mite extracts containing 10 μ g/mL Der p1 in sensitized animals pretreated with saline produced an increase in airway responsiveness to methacholine, as determined by an increase in the average Penh values compared with naive mice (Fig 3, G). This hyperresponsiveness was drastically prevented by vaccinations with ProDer p1 *coli* ($P < .05$) but not with ProDer p1 CHO/alum ($P > .05$).

DISCUSSION

Although we and others successfully produced a correctly folded recombinant ProDer p1 in insect, animal, or *P. pastoris*,^{10-12, 22,23} the design of Der p1-based hypoallergens remains poorly investigated. To our knowledge, aggregated recombinant ProDer p1 obtained by heat denaturation constitutes the only full-length Der p1 variant showing drastically reduced IgE reactivity.¹⁸

Attempts to express mature Der p1 in *E. coli* have been unsuccessful^{24,25} probably because of the absence of the prosequence that plays the role for chaperonin.²⁶ Recombinant ProDer f1, the ProDer p1 homolog from *Dermatophagoides farinae*, was produced in bacteria as inclusion bodies and could be renatured after denaturation/refolding steps.²⁷ Moreover, this Der f1 zymogen could be matured to give enzymatically active Der f1 with IgE reactivity. However, during the renaturation process, a large part of soluble ProDer f1 remained aggregated. This Der f1 form likely displays weak IgE reactivity because it was clearly demonstrated that group 1 mite allergens display strictly conformational IgE binding epitopes.²⁸

Consequently, we produced in the current study ProDer p1 in *E. coli*. As described for ProDer f1, ProDer p1 was highly expressed as inclusion bodies. After purification under denatured conditions, several refolding protocols were tested to renature

recombinant Der p 1, including the use of refolding agents (oxidized/reduced glutathione, L-arginine) and several refolding methods (dialysis, dilution of purified recombinant allergen or renaturation during purification on immobilized Ni²⁺ columns). Three different approaches were used to characterize the folding of ProDer p 1 in native conditions: size exclusion chromatography, FTIR spectroscopy, and anti-Der p 1 ELISA. Under all the refolding conditions tested, recombinant ProDer p 1 remained in native conditions under an aggregated form.

Strikingly, direct ELISA, competition as well as RBL assays demonstrated that the IgE reactivity of aggregated ProDer p 1 was drastically reduced compared with that of the corresponding correctly folded form produced in CHO cells. It must be pointed out that the weaker mediator release activity of ProDer p 1 CHO confirmed that the prosequence domain more likely masks some important IgE binding epitopes, as suggested by Takai et al.¹⁵

The loss of allergenic activity was associated with an alteration of the native-like structure of ProDer p 1 as shown by infrared spectroscopy, confirming that Der p 1 displays conformational IgE epitopes. Together with the maintenance of the Der p 1 T-cell reactivity, we could consider that ProDer p 1 *coli* represents a hypoallergenic variant of Der p 1. We speculate that prokaryotes would tend to express incorrectly folded hypoallergenic variants when the allergen displays complex structural motifs such as disulfide bridges and enzymatic active sites.

It is noteworthy that, by its high molecular weight, aggregated ProDer p 1 *coli* closely resembles recombinant allergen oligomers²⁹ (genetically engineered recombinant proteins expressed as dimers and trimers) as well as allergoids³⁰ (chemical modified high-molecular-weight allergen polymers). Such modified allergens were shown to display reduced reactivity with IgE and represent novel hypoallergenic vaccine prototypes for the treatment of allergy.³¹

The recombinant production of allergen aggregates is more advantageous than allergoids because no additional treatments are needed to disrupt the IgE binding epitope repertoire after allergen purification. Moreover, allergoids are derived from poorly defined allergen extracts, and chemical modifications to reduce their allergenic activity are difficult to control.

We next compared in mice the prophylactic vaccine potential of ProDer p 1 *coli* with that of ProDer p 1 produced in CHO cells. It must be pointed out that control mice were vaccinated with saline because previous animal experiments clearly indicated that control vaccination with alum alone or with Der p 1/alum, respectively, had no effect or amplified the T_H2 allergic response induced after Der p 1/alum sensitization (data not shown). Contrary to monomeric ProDer p 1, the corresponding aggregated allergen developed a specific T_H1-biased immune response characterized by the production of specific IgG_{2a} and the absence of allergen-specific IgE antibodies. Moreover, this T_H1 polarization was maintained after the sensitization by multiple injections of Der p 1 adjuvanted with alum and challenges by exposures to aerosolized mite extract. Indeed, whereas the specific IgG_{2a} response persisted after the sensitizations and the challenges, specific IgE antibodies were detected in ProDer p 1 *coli*-vaccinated mice, but this production was much weaker than that measured in ProDer p 1 CHO-vaccinated and allergic control groups. The cytokine pattern of T-cell proliferation confirmed the T_H1-inducer character of ProDer p 1 *coli* because the drastic reduction of IL-5 production was accompanied by an enhanced IFN- γ secretion. Similar results were previously observed with heat-denatured ProDer p 1 vaccinations.¹⁸

Finally, we could show that ProDer p 1 *coli* immunization efficiently reduced the infiltration of eosinophils in the BAL fluid and prevented airway hyperresponsiveness (AHR) after methacholine challenge in sensitized mice. These results suggest that ProDer p 1 *coli*-based specific immunotherapy could be efficient for the prevention of allergen-induced airway inflammation. It must be pointed out that vaccinations with ProDer p 1 CHO reduced similarly the airway eosinophilia but did not influence the AHR. The AHR results obtained with the ProDer p 1 vaccinations could likely be explained by a net reduction of IL-5 secretion in ProDer p 1 *coli*-vaccinated mice because it is well recognized that development of AHR requires IL-5.³²

Because it was assumed that IL-5 also plays a prominent role in the induction of eosinophil infiltration in the airways,³³ the absence of eosinophil in BAL fluids from ProDer p 1 CHO-vaccinated mice was surprising, considering the strong induction of IL-5 in this group. IgE-dependent and IgE-independent mechanisms can manage the recruitment of eosinophils in the airways.^{34,35} Notably, the IgE-dependent mast cell activation is important in airway eosinophil infiltration because release of mediators from activated mast cells contributes to eosinophil recruitment, activation, and survival.^{36,37} Because it was previously demonstrated that mature Der p 1 displays more IgE reactivity than ProDer p 1, the weaker IgE binding capacity of the proform could lead to a reduction of the IgE-dependent mast cell activation in ProDer p 1 CHO-vaccinated mice after aerosol exposures, explaining, at least partly, the failure of ProDer p 1 CHO-vaccinated mice to develop airway eosinophilia.

The mechanism behind protective vaccination with ProDer p 1 *coli* is not clear but is believed to involve modulation of the B-cell and T-cell responses to the allergen.

ProDer p 1 *coli* aggregation could likely mask the IgE binding epitopes or, alternatively, the improper folding of ProDer p 1 *coli* (confirmed by FTIR) could induce the loss of IgE binding epitopes, both leading to the absence of the IgE-mediated antigen presentation. Modified allergens, lacking B-cell epitopes, use phagocytic or pinocytic antigen-uptake mechanisms through monocytes, macrophages, or dendritic cells, bypassing IgE cross-linking and IgE-mediated antigen presentation in mast cells, basophils, Langerhans cells, eosinophils, and epidermal cells. This effect induces a balanced T_H0 or T_H1-like cytokine pattern by T cells, resulting in a lower IgE and higher IgG production by memory B cells.^{38,39}

In conclusion, we show for the first time that an aggregated recombinant Der p 1 derivative can prevent the development of mite allergy. We consider aggregated ProDer p 1 as a promising candidate for use in allergen-specific immunotherapy to prevent allergy or to treat patients with allergy. The evaluation of the hypoallergenic vaccine in the treatment of Der p 1-sensitized mice is currently under study.

Clinical implications: Hypoallergenic variant of the mite allergen Der p 1 is an attractive vaccine candidate against allergy.

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